



**OXIDANT SCAVENGERS FOR TREATMENT OF  
DIABETES OR USE IN TRANSPLANTATION OR  
INDUCTION OF IMMUNE TOLERANCE**

TECHNICAL FIELD

The present invention relates, in one embodiment, to a method of preventing or treating diabetes using low molecular weight antioxidants. In a further embodiment, the invention relates to a method of protecting and/or enhancing viability of cells/tissues/organs during isolation (harvesting), preservation, expansion and/or transplantation. In yet another embodiment, the present invention relates to a method of inducing immune tolerance. The invention also relates to compounds and compositions suitable for use in such methods.

BACKGROUND

Type I diabetes is caused by the autoimmune destruction of insulin-producing pancreatic  $\beta$  cells. A large body of evidence supports the concept that the antigen-specific, T cell-mediated infiltration of inflammatory cells to the pancreas leads to the generation of reactive oxygen species (ROS) [superoxide, ( $O_2^{\cdot-}$ ), hydroxyl radical ( $\cdot OH$ ), nitric oxide ( $NO\cdot$ ), peroxynitrite ( $ONOO^-$ )], and pro-inflammatory cytokines ( $TNF-\alpha$ ,  $IL-1\beta$  (interleukin  $1\beta$ ) and  $IFN-\gamma$  (interferon  $\gamma$ ) (Rabinovitch et al,

Endocrinology 137:2093-2099 (1996), Mandrup-Poulsen, Diabetologia 39:1005-1029 (1996), Eizirik et al, Diabetologia 39:875-890 (1996), Mandrup-Poulsen et al, Eur. J. Endocrinol. 134:21-30 (1996)). Synergistic interaction between ROS (reactive oxygen species) and these cytokines results in the ultimate destruction of the pancreatic  $\beta$  cells.

Locally produced ROS are involved in the effector mechanisms of  $\beta$  cell destruction (Rabinovitch et al, Endocrinology 137:2093-2099 (1996), Mandrup-Poulsen, Diabetologia 39:1005-1029 (1996), Eizirik et al, Diabetologia 39:875-890 (1996), Grankvist et al, Biochem. J. 182:17-25 (1979), Kroncke et al, Biochem. Biophys. Res. Commun. 175:752-758 (1991), Corbet et al, J. Clin. Invest. 90:2384-2391 (1992)). *In vitro*, T cell and macrophage cytokines such as IFN- $\gamma$ , IL-1 $\beta$  and TNF- $\alpha$  (tumor necrosis factor- $\alpha$ ) induce the production of ROS by  $\beta$  cells. In addition, ROS either given exogenously or elicited in  $\beta$  cells by cytokines lead to  $\beta$  cell destruction (Lortz et al, Diabetes 49:1123-1130 (2000)). This destruction appears to ultimately be caused by an apoptotic mechanism (Kurrer et al, Proc. Natl. Acad. Sci. USA 94:213-218 (1993), O'Brien et al, Diabetes 46:750-757 (1997), Chervonski et al, Cell 89:17-24 (1997), Itoh et al, J. Exp. Med. 186:613-618 (1997)).  $\beta$  cells engineered to over-express antioxidant proteins have been shown to be

resistant to ROS and NO<sup>•</sup> (Grankvist et al, Biochem. J. 199:393-398 (1981), Malaisse et al, Proc. Natl. Acad. Sci. USA 79:927-930 (1982), Lenzen et al, Free Radic. Biol. Med. 20:463-466 (1996), Tiedge et al, Diabetes 46:1733-1742 (1997), Benhamou et al, Diabetologia 41:1093-1100 (1998), Tiedge et al, Diabetes 47:1578-1585 (1998), Tiedge et al, Diabetologia 42:849-855 (1999)). Furthermore, stable expression of manganese superoxide dismutase (Mn-SOD) in insulinoma cells prevented IL-1 $\beta$ -induced cytotoxicity and reduced nitric oxide production (Hohmeier et al, J. Clin. Invest. 101:1811-1820 (1998)). Finally, others have shown that transgenic mice with  $\beta$  cell-targeted over-expression of copper, zinc SOD or thioredoxin are resistant to autoimmune and streptozotocin-induced diabetes (Kubisch et al, Proc. Natl. Acad. Sci. USA 91:9956-9959 (1994), Kubisch et al, Diabetes 46:1563-1566 (1997), Hotta et al, J. Exp. Med. 188:1445-1451 (1998)).

SOD mimics have been designed with a redox-active metal center that catalyzes the dismutation of O<sub>2</sub><sup>-</sup> in a manner similar to the active metal sites of the mammalian Cu, Zn- or Mn- containing SODs (Fridovich, J. Biol. Chem. 264:7761-7764 (1989), Pasternack et al, J. Inorg. Biochem. 15:261 (1981), Faulkner et al, J. Biol. Chem. 269:23471-23476 (1994), Batinic-Haberle et al, J. Biol. Chem. 273:24521-24528 (1998), Patel et al, Trends Pharmacol. Sci. 20:359-364 (1999),

Spasojevic et al, Inorg. Chem. 40:726 (2001)). The manganese porphyrins have a broad antioxidant specificity, which includes scavenging  $O_2^-$  (Batinic-Haberle et al, Inorg. Chem. 38:4011 (1999)),  $H_2O_2$  (Spasojevic et al, Inorg. Chem. 40:726 (2001), Day et al, Arch. Biochem. Biophys 347:256-262 (1997)),  $ONOO^-$ , (Ferrer-Sueta et al, Chem. Res. Toxicol. 12:442-449 (1999)),  $NO$  (Spasojevic et al, Nitric Oxide: Biology and Chemistry 4:526 (2000)) and lipid peroxy radicals (Day et al, Free Radic. Biol. Med. 26:730-736 (1999)). SOD mimics have recently been found to rescue vascular contractility in endotoxic shock (Zingarelli et al, Br. J. Pharmacol. 120:259-267 (1997)), protect neuronal cells from excitotoxic cell death (Patel et al, Neuron 16:345-355 (1996)) and apoptosis (Patel, J. Neurochem. 71:1068-1074 (1998)), inhibit lipid-peroxidation (Day et al, Free Radic. Biol. Med. 26:730-736 (1999), Bloodsworth et al, Free Radic. Biol. Med. 28:1017-1029 (2000)), block hydrogen peroxide-induced mitochondrial DNA damage (Milano et al, Nucleic Acids Res. 28:968-973 (2000)), and partially rescue a lethal phenotype in a manganese superoxide dismutase knockout mouse (Melov et al, Nat. Genet. 18:159-163 (1998)). The ability of the SOD mimics to scavenge a broad range of ROS allows for their utilization in inflammatory diseases.

The present invention provides a pharmacological approach to protect  $\beta$  cells from the T cell mediated

ROS and cytokine destruction associated with autoimmune diabetes by employing a synthetic metalloporphyrin-based superoxide dismutase mimic. The invention also provides a method of improving survival of pancreatic  $\beta$  islet cells following transplantation.

#### SUMMARY OF THE INVENTION

The present invention relates, in one embodiment, to a method of preventing or treating diabetes using low molecular weight antioxidants. In a further embodiment, the invention relates to a method of protecting and/or enhancing viability of cells/tissues/organs during isolation (harvesting), preservation, expansion and/or transplantation. In yet another embodiment, the present invention relates to a method of inducing immune tolerance. The invention also relates to compounds and compositions suitable for use in such methods.

Objects and advantages of the present invention will be clear from the description that follows.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B. SOD mimic administration delays or prevents T cell-mediated diabetes in young NOD (nonobese diabetic).*scid* (severe combined immunodeficient) recipients after diabetogenic T cell clone BDC-2.5 transfer. Fig. 1A. NOD.*scid* mice 9-14 days

of age were injected i.p., one day prior to adoptive transfer of  $1 \times 10^7$  BDC-2.5 T cell clones, with 10 mg/kg of the SOD mimic or HBSS control. The SOD mimic was then given every other day for a total of 5 days. The data represented in Fig. 1A is the combination of 3 separate experiments. Fig. 1B. Representative pancreatic histology from young NOD.scid mice treated with SOD mimic or control after adoptive transfer of the T cell clone BDC-2.5. Fig. 1Ba. Hematoxylin and Eosin (H&E) staining of a heavily infiltrated pancreas from the positive control, a young NOD.scid mouse after adoptive transfer of BDC-2.5. Fig. 1Bb.,c. H & E staining of pancreas from young NOD.scid treated with SOD mimic (10mg/kg) after adoptive transfer of BDC-2.5. Fig. 1Bd. Aldehyde-fuchsin (A/F) staining of pancreas from SOD mimic-treated NOD.scid mouse.

Figures 2A-2C. Production of IFN- $\gamma$  by BDC-2.5 treated cells with SOD mimic *in vitro* using three types of T cell stimulation. Fig. 2A. 96-well round bottom plates were pre-coated with 0.125 $\mu$ g/ml  $\alpha$ -CD3 and 1  $\mu$ g/ml  $\alpha$ -CD28 for 1 hr at 37°C. The plates were washed twice with sterile HBSS and then blocked with complete medium (CM) at 37°C for 1 hour. Blocking solution was removed and  $2 \times 10^4$  BDC-2.5 T cell clones were added to the wells in the presence or absence of the SOD mimic at concentrations of 34 and 17  $\mu$ M; the

negative control was BDC-2.5 without  $\alpha$ -CD3 and  $\alpha$ -CD28. Cultures were incubated at 37°C for 48 hr before the supernatants were harvested and assayed by sandwich ELISA for IFN- $\gamma$  production. Data are the mean and SEM of 3 separate experiments; p values are shown for conditions where statistical significance was noted. Fig. 2B. BDC-2.5 T cells were plated at  $2 \times 10^4$  cells/well in 96-well flat-bottom plates with or without  $5 \times 10^5$  irradiated syngeneic spleen cells as APC (antigen presenting cell/s) and Con A ( $2.5 \mu\text{g/ml}$  final concentration), in the presence or absence of the SOD mimic at concentrations of  $34 \mu\text{M}$  and  $17 \mu\text{M}$ . Cultures were incubated at 37°C for 24 hr before the supernatants were harvested and assayed by sandwich ELISA for IFN- $\gamma$  production. Data are the mean and SEM of 3 separate experiments. Fig. 2C. BDC-2.5 T cell clones were cultured in 96-well flat-bottom plates at a density of  $2 \times 10^4$  cells/well, with 5000 islet-cells as antigen and  $2.5 \times 10^4$  APC, in the presence or absence of SOD mimic at 34 and  $17 \mu\text{M}$ . Cultures were incubated at 37°C for 48 hr before the supernatants were harvested and assayed by sandwich ELISA for IFN- $\gamma$  production. Data are the mean and SEM of 3 separate experiments.

Figure 3. *In vivo* treatment of 2.5 TCR Tg (transgenic)/NOD mice with the SOD mimic. 2.5 TCR-Tg/NOD mice were treated for 7 days with 10 mg/ml SOD

mimic or HBSS. Spleen cells were harvested from the animals on day 8 and the T cells were purified from SOD mimic or control mice and plated ( $6 \times 10^4$  cells/well) with APC ( $3 \times 10^5$  cell/well) from either SOD mimic or control mice in a criss-cross fashion. The cultures were pulsed with  $1 \mu\text{M}$  of HRPI-RM peptide and on day 4 of culture, the plate was pulsed with ( $1\mu\text{Ci } ^3\text{H-TdR}$ ) for 6 hr before harvest. Values are the mean and SEM of triplicate wells. Data are representative of duplicate experiments.

Figures 4A and 4B. LPS (lipopolysaccharide)-induced respiratory burst and cytokine production by peritoneal macrophages. Fig. 4A. Peritoneal macrophages (PC) were harvested from unprimed NOD mice and plated ( $5 \times 10^5$  cells/well) in 24-well plates in CM with *E. coli* LPS (055:B5) at 200 ng/ml in the presence or absence of the SOD mimic at  $34 \mu\text{M}$  or  $3.4 \mu\text{M}$  final concentration. Cultures were incubated at  $37^\circ\text{C}$  for 48 hr; the cells were trypsinized, and washed to remove the trypsin, and subsequently transferred to microfuge tubes. PMA was added to a final concentration of 50 ng/ml. After incubation at  $37^\circ\text{C}$  for 20 min, superoxide production was assessed spectrophotometrically by ferricytochrome c reduction using an  $\epsilon = 20,000 \text{ M}^{-1} \text{ cm}^{-1}$ . The reduction was monitored over a period of 10 min. Data are mean and SEM or triplicate wells and representative of



duplicate experiments. Fig. 4B. Peritoneal macrophages were harvested from unprimed NOD mice by washing the cavity of each animal with 7 ml of HBSS. The cells were then washed 2x in sterile HBSS and adjusted to  $5 \times 10^5$  cells/well in a 24 well plate in CM with *E. coli* LPS (05:B5) at 200 ng/ml in the presence or absence of 34  $\mu$ M or 17  $\mu$ M SOD mimic. Cultures were incubated at 37°C for 48 hr before the supernatants were harvested and assayed by specific sandwich ELISA for TNF- $\alpha$ . The data are the mean and SEM of 3 separate experiments.

Figures 5A and 5B. Alloxan and cytokine cytotoxicity of SOD mimic treated NIT-1 cells. Fig. 5A. NIT-1 cells were grown to confluence in 12-well tissue culture dishes. Media was removed and replaced with PBS alone or PBS containing 34  $\mu$ M SOD mimic. All solutions were supplemented with 4% FCS. After 1 hour incubation, 10 mM alloxan was added to the appropriate wells, and cells were incubated for an additional 2 hours. Cells were washed, collected by trypsinization and processed for viability via ethidium bromide/acridine orange fluorescence  $\square$  live,  $\square$  live apoptotic. Data are representative of duplicate experiments. Fig. 5B. NIT-1 cells were grown to 80% confluence in 12-well plastic tissue culture dishes. Growth media was removed and replaced with 500  $\mu$ l/well of either media alone or media + 34  $\mu$ M SOD mimic.

After 1 hour incubation, 500  $\mu$ l/well of media alone, media or 20 ng/ml IL-1 (10 ng/ml final concentration) +/- 34  $\mu$ M SOD mimic were added. Cells were incubated an additional 48 hr, and assessed for viability via ethidium bromide/acridine orange fluorescence  $\square$  live,  $\blacksquare$  live apoptotic. Values are the mean and SEM of triplicate wells per treatment.

Figure 6. Protection from streptozotocin-induced diabetes by *in vivo* treatment with SOD mimetic.

Figure 7. Protection of islet transplants from streptozotocin-induced diabetes by *in vitro* culture with SOD mimetic.

Figure 8. Facilitation of islet engraftment in spontaneously diabetic NOD mice by *in vitro* pre-treatment with SOD mimetic.

Figure 9. Structures of prophyrins mimetics.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods of preventing or treating diabetes using low molecular weight antioxidants (eg mimetics of scavengers of reactive oxygen species, including mimetics of SODs, catalases and peroxidases). In accordance with the

invention, the present mimetics can be used to prevent, delay the onset of and/or limit the severity of diabetes resulting, for example, from the death of pancreatic islet cells due to autoimmune diseases or free radical induced toxicity, or toxins or drugs the effects of which are mediated by free radical damage. The invention further relates to a method of enhancing cell survival (for example,  $\beta$  islet cell survival) following transplantation. The invention further relates to formulations suitable for use in such methods.

Mimetics of scavengers of reactive oxygen species appropriate for use in the present methods include methine (ie meso) substituted porphines and substituted tetrapyrroles, or pharmaceutically acceptable salts thereof (eg chloride or bromide salts). The invention includes both metal-free and metal-bound porphines and tetrapyrroles. In the case of metal-bound porphines and tetrapyrroles, manganic derivatives are preferred, however, metals other than manganese such as iron (II or III), copper (I or II), cobalt (II or III), or nickel (I or II), can also be used. It will be appreciated that the metal selected can have various valence states, for example, manganese II, III, IV or V can be used. Zinc (II) can also be used even though it does not undergo a valence change and therefore will not directly scavenge superoxide. The choice of the metal can affect

selectivity of the oxygen species that is scavenged. Examples of such mimetics are shown in Figure 9 and/or are described in USPs 5,994,339 and USP 6,127,356 and in U.S. Application Nos. 09/184,982, 09/296,615, 09/490,537 and 60/211,857 (these applications are incorporated in their entirety by reference). Appropriate methods of synthesis are described in these patents and applications.

In addition to the mimetics described in the above-identified patents and applications, manganese salen compounds can also be used (Baudry et al, Biochem. Biophys. Res. Commun. 192:964 (1993)). Manganese macrocyclic complexes, such as those described by Riley et al (Inorg. Chem. 35:5213 (1996)), Deune et al (Plastic Reconstr. Surg. 98:712 (1996)), Lowe et al (Eur. J. Pharmacol. 304:81 (1996)) and Weiss et al (J. Biol. Chem. 271:26149 (1996)) can also be used. (See also USP 6,084,093, 5,874,421, 5,637,578 and 5,610,293.)

The compounds of the invention can be used alone or in combination with other agents to induce immune tolerance. As shown in the Examples that follow, the present mimetics can be used to alter events that occur during antigen presentation to lymphocytes to create a condition of immune tolerance. This process can be used in the treatment of diseases involving reaction to foreign antigens (e.g., transplantation) or self antigens (e.g., autoimmune diseases such as

diabetes, multiple sclerosis, glomerulonephritis, rheumatoid arthritis and collagen vascular diseases).

The mimetics of the invention (including those in Fig. 9) can also be used to protect or enhance viability of cells/tissues/organs, e.g., mammalian cells/tissues/organs, including stem cells, pancreatic  $\beta$  cells, liver progenitor cells, and progenitor cells isolated from adult tissue harvested from cadavers. The mimetics can be used during the processes of isolation (harvesting), preservation (e.g., freezing and thawing (or "cryopreservation" which encompasses both freezing and thawing)), expansion and/or transplantation. Cells/tissues/organs treated with the present mimetics show enhanced potential in transplantation therapy. Specifically, cells/tissues/organs treated with the mimetics can be used in transplant therapy to treat, for example, diabetes, liver failure, and inherited metabolic conditions. Cells/tissues/organs used in such therapies (particularly treatment of metabolic disorders) can be genetically engineered. (By way of example, it is noted that AEOL 10112 has been used in connection with hepatic progenitors. In this regard, the cryopreservation buffer can be supplemented with trace elements (selenium ( $10^{-3}M$ ), copper ( $10^{-7}M$ ), zinc ( $5 \times 10^{-11}M$ )) and an antioxidant (e.g., a porphyrin SOD mimetic at 10mcg/ml; ascorbate acid, used at about 0.1mg/ml)).

Further, the mimetics of the invention (including those in Fig. 9) can be used to protect cells/tissue/organs from toxicity, including free radical induced toxicity, during harvesting, preservation and transport. For example, livers, hearts and kidneys for transplant can be treated with the present mimetics.

The compounds described above, metal bound and metal free forms, can be formulated into pharmaceutical compositions suitable for use in the present methods. Such compositions include the active agent (mimetic) together with a pharmaceutically acceptable carrier, excipient or diluent. The composition can be present in dosage unit form for example, tablets, capsules or suppositories. The composition can also be in the form of a sterile solution, e.g., a solution suitable for injection (e.g., subcutaneous, i.p. or i.v.) or nebulization. Compositions can also be in a form suitable for ophthalmic use. The invention also includes compositions formulated for topical administration, such compositions taking the form, for example, of a lotion, cream, gel or ointment. The concentration of active agent to be included in the composition can be selected based on the nature of the agent, the dosage regimen and the result sought. The compounds can also be encapsulated in lysosomes and thereby targeted to enhance delivery.

The dosage of the composition of the invention to be administered can be determined without undue experimentation and will be dependent upon various factors including the nature of the active agent (including whether metal bound or metal free), the route of administration, the patient, and the result sought to be achieved. A suitable dosage of mimetic to be administered IV or topically can be expected to be in the range of about 0.01 to 50 mg/kg/day, preferably, 0.1 to 10 mg/kg/day, more preferably 0.1 to 6 mg/kg/day. For aerosol administration, it is expected that doses will be in the range of 0.001 to 5.0 mg/kg/day, preferably, 0.01 to 1 mg/kg/day. Suitable doses will vary, for example, with the compound and with the result sought. The concentration of mimetic presentation in a solution used to treat cells/tissues/organs in accordance with the methods of the invention can also be readily determined and will vary with the mimetic, the cell/tissue/organ and the effect sought.

Certain aspects of the invention can be described in greater detail in the non-limiting Example that follows.

## EXAMPLE 1

### Inhibition of Autoimmune Diabetes by Metalloporphyrin-Based Superoxide Dismutase

#### Experimental Details

##### Mice

NOD.scid breeding pairs were obtained either from The Jackson Laboratory (Bar Harbor, ME) or the breeding colony at the Barbara Davis Center. NOD, NOD.scid, and BDC-2.5-TCR-Tg/NOD (2.5 TCR Tg/NOD) mice were bred and housed under specific pathogen-free conditions in the Center for Laboratory Animal Care (CLAC) at the University of Colorado Health Sciences Center.

##### Expansion cultures of BDC-2.5

Expansion cultures for *in vivo* transfers were produced by culturing  $3-6 \times 10^6$  T cells from 4-day restimulation cultures (Haskins et al, Diabetes 37:1444-1448 (1988), Haskins et al, Proc. Natl. Acad. Sci. USA 86:8000-8004 (1989)) in 60 ml complete medium (CM) and 14 U/ml IL-2. CM is DMEM supplemented with 44 mM sodium bicarbonate, 0.55 mM L-arginine, 0.27 mM L-asparagine, 1.5 mM L-glutamine, 1 mM sodium pyruvate, 50 mg/L gentamicin sulfate, 50  $\mu$ M 2-ME, 10 mM HEPES, and 10% FCS. Cells were cultured in 75-cm<sup>2</sup> flasks for 4 days at 37°C and 10% CO<sub>2</sub>. T cells were harvested,



washed three times, resuspended in HBSS, and injected into young (< 15 days of age) NOD.scid recipients.

Metalloporphyrin superoxide dismutase mimic  
(MnTE2PyP5<sup>+</sup>)

The SOD mimic Mn(III) tetrakis(*N*-ethylpyridinium-2-yl)porphyrin (MnTE2PyP5<sup>+</sup>) (AEOL 10113) (SOD mimic) was obtained from Incara Pharmaceuticals. Stock solutions of 600 µg/ml in sterile HBSS for *in vivo* use, or 680 µM in sterile CM for *in vitro* experiments were prepared.

Adoptive transfer of BDC-2.5 T cell clones

Experimental mice were young NOD.scid mice 3-14 days of age. The recipient mice were given one i.p. injection with BDC-2.5 ( $1 \times 10^7$  cells) 1 day after the administration of either the SOD mimic or HBSS as a control. The mimic or HBSS was administered every other day for a total of five treatments. Urine glucose was monitored daily and when animals became diabetic, blood glucose measurements were taken. Overt diabetes was defined as a positive urine glucose (>1%), followed by a positive blood glucose test of >250 mg/dl (14 mM). Recipients were sacrificed when blood glucose readings were 320 mg/dl (18 mM) or higher. At sacrifice, the pancreata were removed for histological analysis.

## Histology

At sacrifice, pancreata were removed and placed in formalin for at least 24 hr. Pancreata were subsequently embedded in paraffin, sectioned, and stained with hematoxylin-eosin (H&E) to detect mononuclear cell infiltration or aldehyde fuchsin (A/F) to detect insulin.

## Preparation of purified CD4<sup>+</sup> T cells from 2.5 TCR-Tg/NOD mice

2.5 TCR-Tg/NOD mice were injected i. p. with either 10 mg/kg SOD mimic or HBSS every day for 7 days. At day 8, animals were sacrificed, and the spleens were removed for isolation of CD4<sup>+</sup> T cells by immunomagnetic positive selection using the MACs magnetic cell separation kit (Miltenyi Biotec, Auburn CA) according to the manufacturer's protocol. The purified T cells were then plated in 96-well round-bottom plates, pre-coated with 50  $\mu$ l of a 1  $\mu$ M solution of a BDC-2.5 peptide mimotope, HRPI-RM, as antigen. Antigen-presenting (APC), treated with either the SOD mimic or HBSS, were added to the T cells in a crisscross fashion. The assay plates were incubated for 4 days, and then pulsed with 1  $\mu$ Ci of <sup>3</sup>H-TdR for 6 hrs before harvesting.

## T cell and macrophage functional assays

IFN- $\gamma$  production by BDC 2.5 was assessed by sandwich ELISA analysis of responder T cells stimulated with  $\alpha$ -CD3 and  $\alpha$ -CD28, Con-A or islet cell antigen. For  $\alpha$ -CD3 /  $\alpha$ -CD28 stimulation, 96-well round bottom plates were pre-coated with 0.125  $\mu$ g/ml  $\alpha$ -CD3 and 1  $\mu$ g/ml  $\alpha$ -CD28 for 1 hr at 37°C. After washing the plates with sterile HBSS and blocking with CM at 37°C for 1 hr, the blocking solution was removed and the BDC-2.5 T cell clone ( $2 \times 10^4$  cells) was added to the wells in the presence or absence of the SOD mimic at concentrations of 17 and 34  $\mu$ M. The negative control was BDC-2.5 alone without  $\alpha$ -CD3 and  $\alpha$ -CD28. For Con-A stimulation, BDC-2.5 T cells were plated at  $2 \times 10^4$  cells/well in 96-well flat-bottom plates with or without  $5 \times 10^5$  irradiated syngeneic spleen cells as APC and Con A (2.5  $\mu$ g/ml final concentration), in the presence or absence of the SOD mimic at concentrations of 17 and 34  $\mu$ M. Cultures were incubated at 37°C for 24 hr before the supernatants were harvested and assayed by sandwich ELISA for IFN- $\gamma$  production. For antigen-specific recall assays, BDC-2.5 T cells were cultured in 96-well flat-bottom plates at a density of  $2 \times 10^4$  cells/well, with 5000 islet-cells as antigen and  $2.5 \times 10^4$  APC, in the presence or absence of 17 and 34  $\mu$ M SOD mimic. Cultures were incubated at 37°C for 48 hr before the supernatants were harvested and assayed for IFN- $\gamma$ . For macrophage assays, peritoneal macrophages (PC) were harvested from unprimed NOD mice

by lavage, washed 2x in sterile HBSS, and then adjusted to  $5 \times 10^5$  cells/well in a 24-well plate in CM with *E. coli* LPS (055:B5) at 200 ng/ml in the presence or absence of 17 or 34  $\mu$ M SOD mimic. Cultures were incubated at 37°C for 48 hr before the supernatants were harvested and assayed by specific sandwich ELISA for TNF- $\alpha$  production, following the manufacturer's protocol (R&D Systems). The remaining cells were collected by trypsinization, and washed 3x in sterile PBS and 4% FCS.

#### Respiratory burst of peritoneal macrophages

Peritoneal macrophages (PC), harvested as described above, were washed 2x in sterile HBSS and then plated ( $5 \times 10^5$  cells/well) in 24-well plates in CM medium with *E. coli* LPS (055:B5) at 200 ng/ml in the presence or absence of the SOD mimic at 34 or 3.4  $\mu$ M. Cultures were incubated at 37°C for 48 hr. Cells were trypsinized and then washed to remove the trypsin and subsequently transferred to microfuge tubes. PMA was added to a final concentration of 50 ng/ml. After incubation at 37°C for 20 min, superoxide production was assessed spectrophotometrically by ferricytochrome c reduction using an  $\epsilon = 20,000 \text{ M}^{-1}\text{cm}^{-1}$ , monitoring the reduction over a period of 10 min.

#### Determination of beta cell apoptosis

*In vitro* apoptosis studies were conducted using the  $\beta$ -cell adenoma line NIT-1 (Hamaguchi et al, Diabetes 40:842-849 (1991)). Tumor cells were propagated in 75 cm<sup>2</sup> flasks at 37° C in CM. Cell lines were re-fed with new medium every other day and were grown to confluence in the 75 cm<sup>2</sup> tissue culture flasks, at which time they were harvested using non-enzymatic Cell Dissociation Buffer (Gibco, BRL; Grand Island, NY) and transferred to the appropriate culture dishes for either expansion or for the experiments described. Alloxan monohydrate (Sigma) was prepared fresh as a 0.5 M stock solution in PBS adjusted to pH 2 with hydrochloric acid. IL-1 $\beta$  was purchased from R & D Systems (Minneapolis MN). NIT-1 cells were grown to confluence in 12-well tissue culture dishes. Media was removed and replaced with PBS alone or PBS containing 34  $\mu$ M mimic. All solutions were supplemented with 4% FCS. After 1 hr incubation, 10 mM alloxan was added to the appropriate wells, and cells were incubated for an additional 2 hr. For cytokine cytotoxicity assays NIT-1 cells were grown to 80% confluence in 12-well plastic tissue culture dishes. Growth media was removed and replaced with 500  $\mu$ l/well of either media alone or media + 34  $\mu$ M mimic. After 1 hr incubation, 500  $\mu$ l/well of media alone, or 20 ng/ml IL-1 (10 ng/ml final concentration) +/- 34  $\mu$ M SOD mimic were added. Cells were incubated an additional 48 hr, and processed. Alloxan or cytokine-treated NIT-1 cells

were harvested by brief trypsinization (200  $\mu$ l/well of a 12-well dish) followed by addition of 50  $\mu$ l FCS to inhibit trypsin. Cells were transferred to a microcentrifuge tube and centrifuged for 5 min at 200 x g. Supernatants were aspirated very carefully, leaving approximately 25  $\mu$ l to allow resuspension of the cell pellets by gentle shaking of the tube. After addition of 1.3  $\mu$ l of dye mix (100  $\mu$ g/ml Acridine Orange + 100  $\mu$ g/ml of EtBr in PBS), 10  $\mu$ l of cell suspension was transferred to a clean microscope slide and a coverslip placed on the suspension. Cells were scored for morphological evidence of apoptosis as described (Squier et al, Assays of Apoptosis. Humana Press, Totowa) using a fluorescence microscope with an excitation of 450-490 nm.

#### Statistical analysis

Statistical significance within experiments was determined using JMP analysis software (SAS Institute, Cary, NC). Survival analysis was done using the product-limit (Kaplan-Meier) method. The endpoint of the experiment was defined as diabetes. Data on animals that did not become diabetic by the end of the experiment were censored. The p values shown were determined by Log-Rank test. All other statistical analysis was done by Oneway analysis of variance Anova (Wilcoxon/ Kruskal-Wallis Rank Sums). If p values were  $\leq$  to 0.05, they were considered significant.

## Results

*In vivo* treatment of young NOD.scid mice with the SOD mimic prevents adoptive transfer of T cell mediated diabetes.

SOD mimic was delivered paraenterally to NOD.scid recipients and 24 hr later, mice were adoptively transferred with the diabetogenic T cell clone BDC-2.5. The SOD mimic or HBSS was then given every other day for a total of 5 treatments. Treatment with the SOD mimic significantly delayed ( $p < 0.0002$ ) onset of diabetes (Fig. 1A), with 50 % of the treated mice still normoglycemic after 28 days at which time all animals were sacrificed for histological examination. Pancreatic tissue from positive control animals (BDC-2.5, no SOD mimic) showed a disseminated infiltrate resembling pancreatitis, and the pancreatic architecture was almost absent (Fig. 1B-a). In contrast, the SOD mimic-treated animals showed an intact pancreatic architecture with few or no infiltrating mononuclear cells (Fig. 1B-b,c), as well as healthy and well-granulated islets (Fig. 1B-d). These data clearly demonstrate that the SOD mimic is inhibiting the infiltration by BDC-2.5 T cells and mononuclear cells to the pancreas. Remarkably, in these experiments, the animals were still protected on day 21, even though the SOD mimic was stopped on day 9, suggesting that this compound prevents priming and subsequent activation of the APC, the T-cell, or both.

Longer administration of the SOD mimic may prove to be even more protective.

Interferon-gamma production by BDC-2.5 is inhibited by the SOD mimic *in vitro*: indirect effect on the APC leading to inhibition of T cell priming.

*In vivo*, BDC-2.5 must be primed by its antigen via presentation by APC in order to become activated and produce IFN- $\gamma$ . Therefore, the SOD mimic could be directly inhibiting T cell activation or the interaction between the APC and the T cell or both. In order to elucidate the mechanism of inhibition of disease transfer, priming of BDC-2.5 was studied *in vitro*, in the presence or absence of APC. To determine if the SOD mimic has a direct effect on IFN- $\gamma$  production by the T cell, BDC-2.5 was cultured with plate bound  $\alpha$ -CD3 and  $\alpha$ -CD28. This type of activation substitutes for both signals 1 and 2 of T cell activation (Mueller et al, J. Immunol. 142:2617-2628 (1989), Mueller et al, Annu. Rev. Immunol. 7:445-480 (1989), Schwartz et al, Cold Spring Harb. Symp. Quant. Biol. 54:605-610 (1989), June et al, Immunology Today 15 (1994)), thus removing the contribution of the APC. Fig. 2A shows that  $\alpha$ -CD3 and  $\alpha$ -CD28 stimulation resulted in no significant difference in IFN- $\gamma$  production by the BDC-2.5 clone, whether or not the SOD mimic was present. These results demonstrate that when plate-bound antibodies substitute for signals 1



and 2, the SOD mimic has no direct effect on the ability of BDC-2.5 to be stimulated to effector function and produce IFN- $\gamma$ . Although primed T cells can directly respond to Con A, optimal Con A-induced T-cell cytokine production requires the participation of accessory cells (e.g., macrophages) (Ahmann et al, J. Immunol. 121:1981-1989 (1978), Hunig et al, Eur. J. Immunol. 13:1-6 (1983), Hunig, Eur. J. Immunol. 13:596-601 (1983), Hunig, Eur. J. Immunol. 14:483-489 (1984), Bekoff et al, J. Immunol. 134:1337-1342 (1985), Roosnek et al, Eur. J. Immunol. 15:652-656 (1985), Hoffmann et al, Lymphokine Res. 5:1-9 (1986)). In order to determine if the SOD mimic could inhibit APC-mediated Con A stimulation of T cells, BDC-2.5 cells were incubated with Con A and APC in the presence or absence of SOD mimic. Fig. 2B shows that 34 or 17  $\mu$ M SOD mimic inhibited IFN- $\gamma$  production by 47 or 30 %, respectively. The levels of IFN- $\gamma$  produced in the presence of the SOD mimic were similar to levels seen when BDC-2.5 was incubated with Con A alone. These results indicate that the SOD mimic inhibits the ability of the APC to optimally stimulate Con A-dependent T cell activation and IFN- $\gamma$  production. To further study the SOD mimic's effect on APC-T cell interactions, IFN- $\gamma$  production was measured in the presence of macrophages as APC and islet cells as a source of antigen. Fig. 2C shows that when this more physiological *in vitro* assay was done, the ability of

BDC-2.5 to make IFN- $\gamma$  was reduced: the 17  $\mu$ M concentration of SOD mimic inhibited by 46% ( $p < 0.05$ ), while the 34  $\mu$ M concentration inhibited by 66% ( $p < 0.05$ ).

*In vivo* treatment of 2.5 TCR Tg/NOD mice with the SOD mimic affects T cell proliferation by inhibiting APC function.

In order to determine if the SOD mimic can influence T cell priming *in vivo*, 2.5 TCR-Tg/NOD mice, which carry the rearranged TCR genes of the BDC-2.5 T cell clone (Katz et al, Cell 74:1089-1100 (1993)), were treated with either the SOD mimic (10 mg/kg) or HBSS each day for 7 days. The T cells and APC were purified from SOD mimic-treated and control mice and cultured in a crisscross proliferation assay using a peptide mimotope HRPI-RM that acts as a stimulating antigen for the 2.5 TCR-Tg cells. Fig. 3 demonstrates that APC from SOD mimic-treated mice showed a reduced ability to support T cell proliferation whether they are presenting the peptide to SOD mimic-treated or untreated T cells. Notably, when control APC were used as presenters, the proliferative response in SOD mimic-treated T cells approached the level achieved with control APC and T cells. These data demonstrate that *in vivo* SOD mimic treatment inhibits the response in TCR-Tg mice primed to a specific self-peptide and indicate that using the SOD mimic in combination with

candidate autoantigens may provide a form of antigen-specific tolerance.

LPS-induced respiratory burst and cytokine production by peritoneal macrophages is inhibited by the SOD mimic.

Macrophages are activated in the two-stage reactions of priming and triggering (Meltzer, J. Immunol. 127:179-183 (1981)). In order to assess the inhibitory effect of the SOD mimic on this process, peritoneal macrophages (PC) were cultured with LPS in the presence or absence of the mimic. The supernatants were collected and the PC (peritoneal macrophages) were washed and triggered with PMA to measure their NADPH oxidase-mediated respiratory burst and superoxide production. Fig. 4A shows that 3.4  $\mu$ M SOD mimic results in a 75% reduction in superoxide production and increasing the concentration of SOD mimic to 34  $\mu$ M did not significantly further decrease superoxide production. Moreover, Fig. 4B shows that TNF- $\alpha$  production by LPS-primed PC was inhibited 34% by 17  $\mu$ M mimic, while 34  $\mu$ M mimic resulted in a 51% inhibition. These data clearly demonstrate that pre-incubation of LPS-primed macrophages with SOD mimic inhibited both activation of NADPH oxidase and TNF- $\alpha$  production. It should be noted that the SOD mimic had been washed off prior to the assay and, therefore, was not present in the extracellular space where

superoxide generation is measured. Therefore, a decrease in superoxide production was not due to the SOD mimic scavenging the extracellular superoxide but rather to a reduction in oxidase-dependent superoxide. The fact that superoxide production by activated macrophages (Fig. 4A) is inhibited by 3.4  $\mu$ M SOD mimic, while inhibition of TNF- $\alpha$  or IFN- $\gamma$  production requires higher SOD mimic concentration (Fig. 4B, 2C), indicates that the oxidant concentration necessary to activate the NADPH oxidase of macrophages is lower than the oxidant concentration necessary to activate the signal transduction pathways required for cytokine production. These results point to the fascinating prospect that biological responses to oxidants are not just "all-or-none", but instead are specific to the pathway involved.

SOD mimic-treated NIT-1 insulinoma cells are protected from alloxan and cytokine-mediated cytotoxicity.

Both alloxan and pro-inflammatory cytokines have been shown to be cytotoxic to  $\beta$ -cells *in vitro*. This series of experiments was designed to determine if the SOD mimic could protect islet cells from alloxan and cytokine-mediated cytotoxicity using the well established NIT-1 insulinoma cell line. Fig. 5A shows that incubation of NIT-1 cells with 10 mM alloxan induces 50% apoptosis compared to 5% for control untreated or control plus SOD mimic. However, NIT-1

cells exposed to alloxan and treated with the SOD mimic show 70% viability.

Fig. 5B demonstrates the protective effect of the SOD mimic on NIT-1 cells exposed to IL-1 $\beta$  in culture. The addition of 10 ng /ml IL-1 $\beta$  was cytotoxic to NIT-1 cells (~50% of the cells were apoptotic) compared to control or control plus SOD mimic. A clear protective effect was seen when NIT-1 cells exposed to IL-1 $\beta$  were treated with SOD mimic. The SOD mimic's protective effect is consistent with other reports of antioxidant proteins conferring resistance to immunological damage in insulinoma cells (Grankvist et al, Biochem. J. 199:393-398 (1981), Malaisse et al, Proc. Natl. Acad. Sci. USA 79:927-930 (1982), Lenzen et al, Free Radic. Biol. Med. 20:463-466 (1996), Tiedge et al, Diabetes 46:1733-1742 (1997), Benhamou et al, Diabetologia 41:1093-1100 (1998), Tiedge et al, Diabetes 47:1578-1585 (1998), Tiedge et al, Diabetologia 42:849-855 (1999)).

## EXAMPLE 2

### Protection from Streptozotocin-induced Diabetes and Facilitation of Islet Engraftment by SOD Mimetic Treatment

1: Protection from streptozotocin-induced diabetes by *in vivo* treatment with SOD mimetic.

Experiment: Diabetes was induced in C57Bl/6 male mice with 160mg/kg streptozotocin (SZ) intravenously. Recipients were either otherwise untreated or were treated with daily intraperitoneal bolus injections with 1mg/kg or 10mg/kg of the SOD mimetic on days -1 through +5 relative to SZ treatment.

Results: 1mg/kg of the mimetic demonstrated some protection from SZ-induced diabetes. Results indicate that the 10mg/kg dose led to protection in 2/5 animals (versus 0/9 in untreated animals). (See Fig. 6.)

2: Protection of islet transplants from streptozotocin-induced diabetes by *in vitro* culture with SOD mimetic.

Experiment: Syngeneic C57Bl/6 islet grafts were pre-treated *in vitro* with SOD mimetic (34 $\mu$ M) for 2 hours and then transplanted in C57Bl/6 challenged with 160mg/kg SZ as described above.

Results: Pre-treatment of the islet graft prior to transplant led to protection in 2/3 islet grafts. (See Fig. 7.)

3: Facilitation of islet engraftment in spontaneously diabetic NOD mice by *in vitro* pre-treatment with SOD mimetic.

Experiment: Recurrence of disease in autoimmune diabetic NOD mice is so vigorous that islet transplants often fail to engraft (i.e., grafts fail

to restore even transient euglycemia). This experiment determined whether initial inflammatory damage to syngeneic NOD islet grafts could be attenuated by treating NOD islets *in vitro* with a SOD mimetic prior to transplant. Syngeneic NOD islet grafts were pre-treated *in vitro* with SOD mimetic (34 $\mu$ M) for 2 hours and then transplanted into spontaneously diabetic (autoimmune) NOD recipient.

Results: The treated NOD islet graft restored euglycemia within 24 hours relative to the untreated control NOD graft that failed to engraft during the initial 5 day observation period. (See Fig. 8)

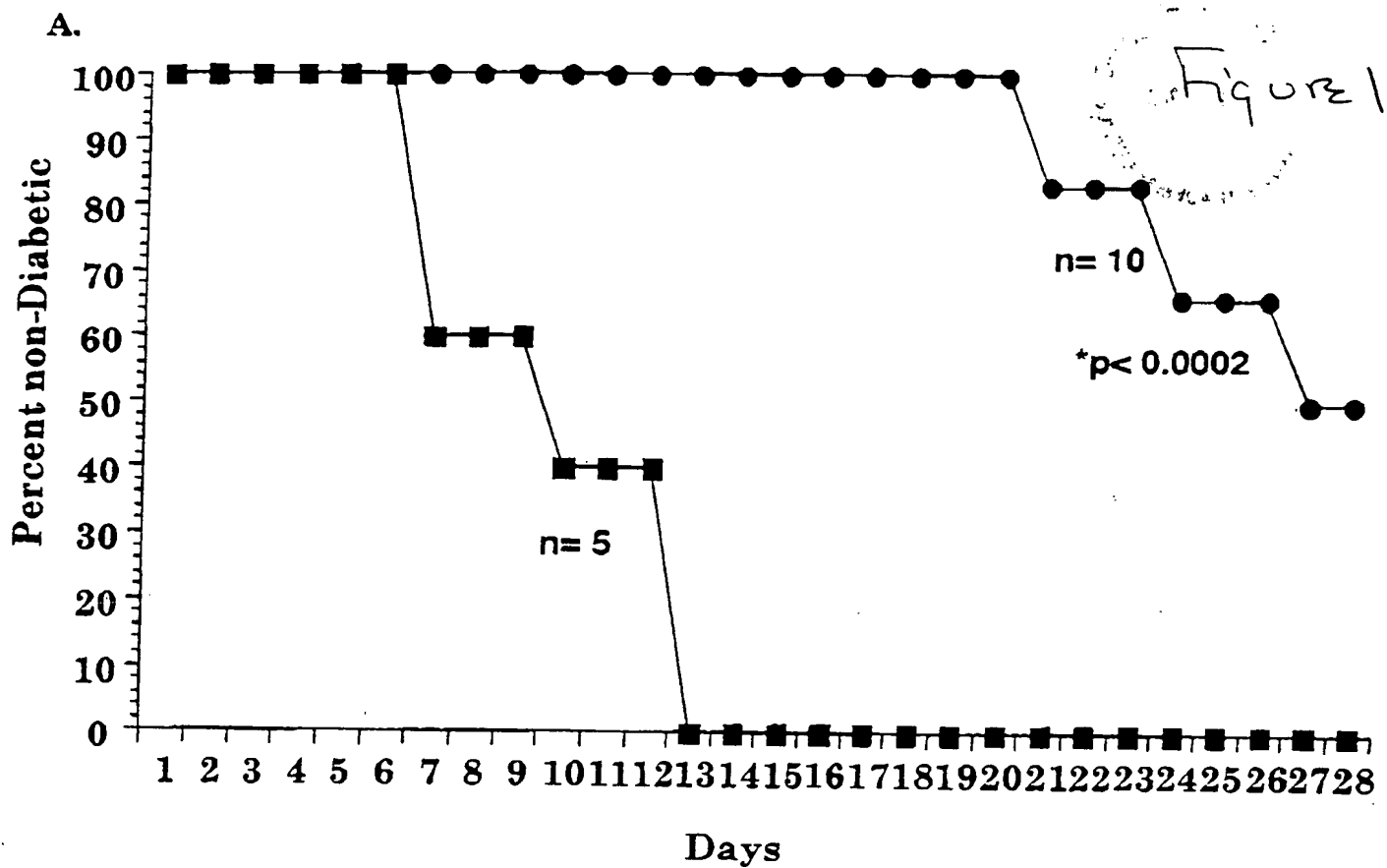
\* \* \*

All documents cited above are hereby incorporated in their entirety by reference.

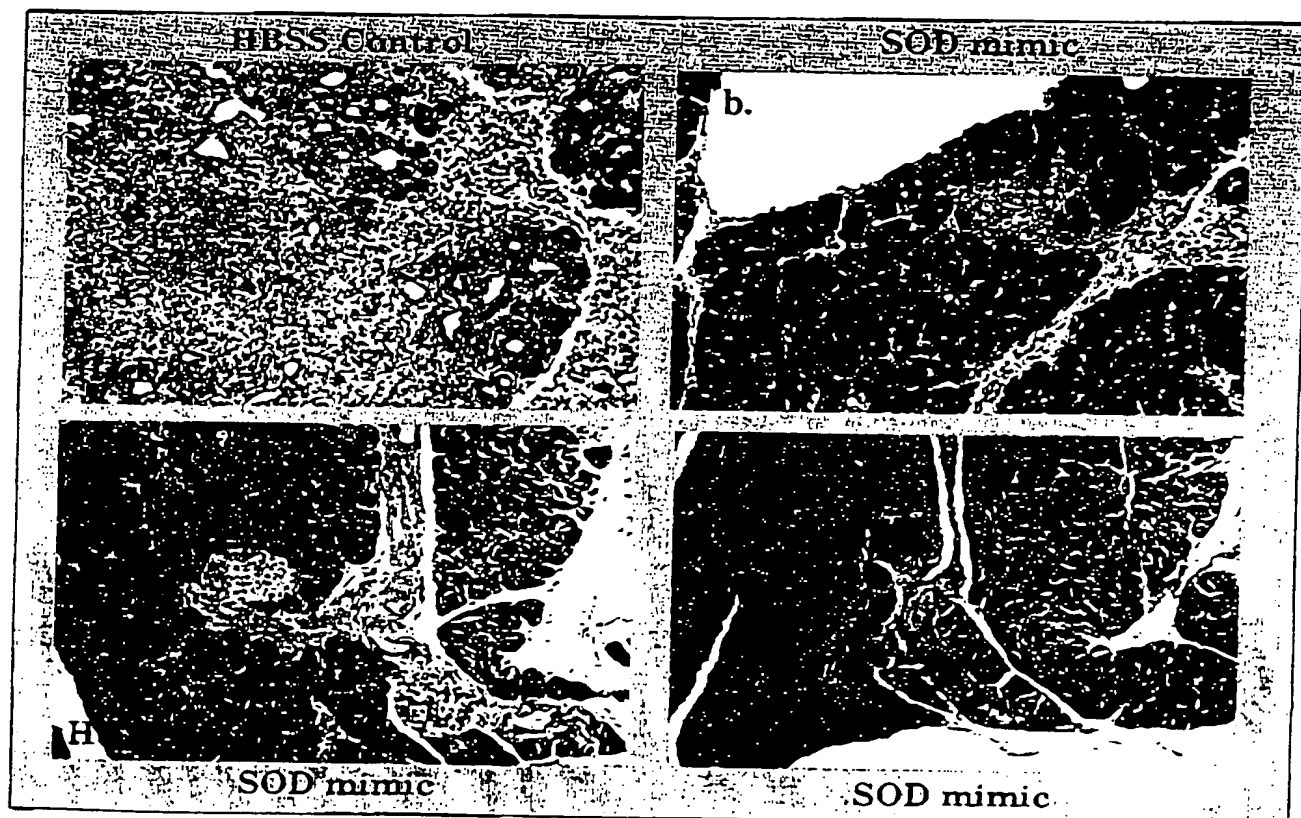
### ABSTRACT

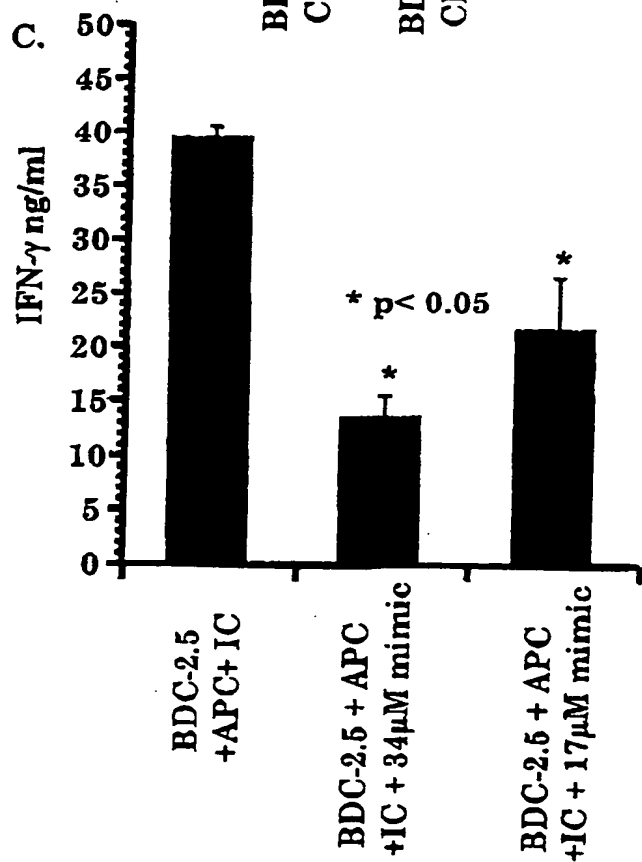
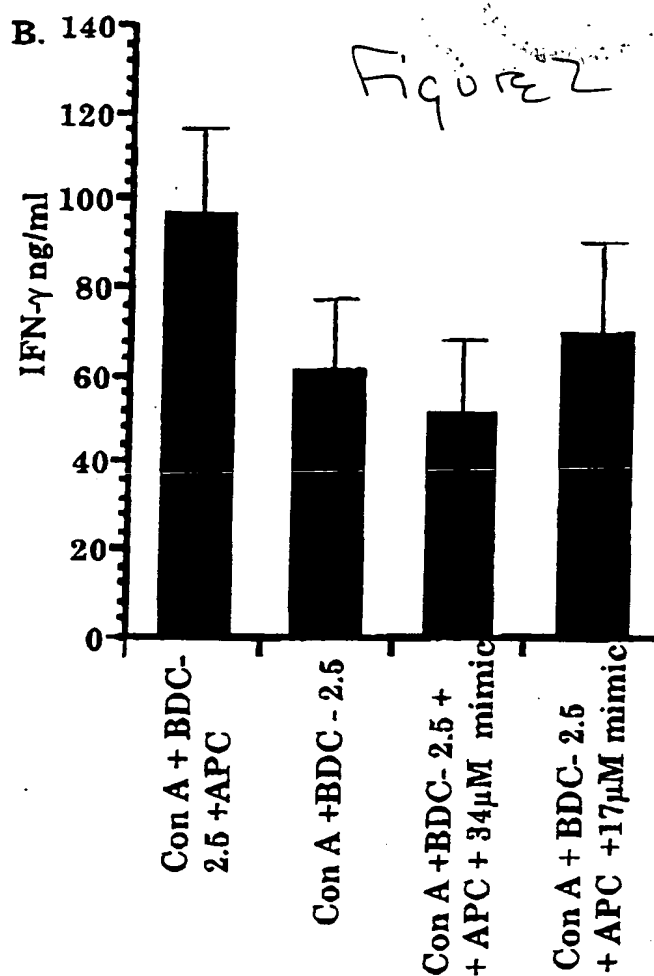
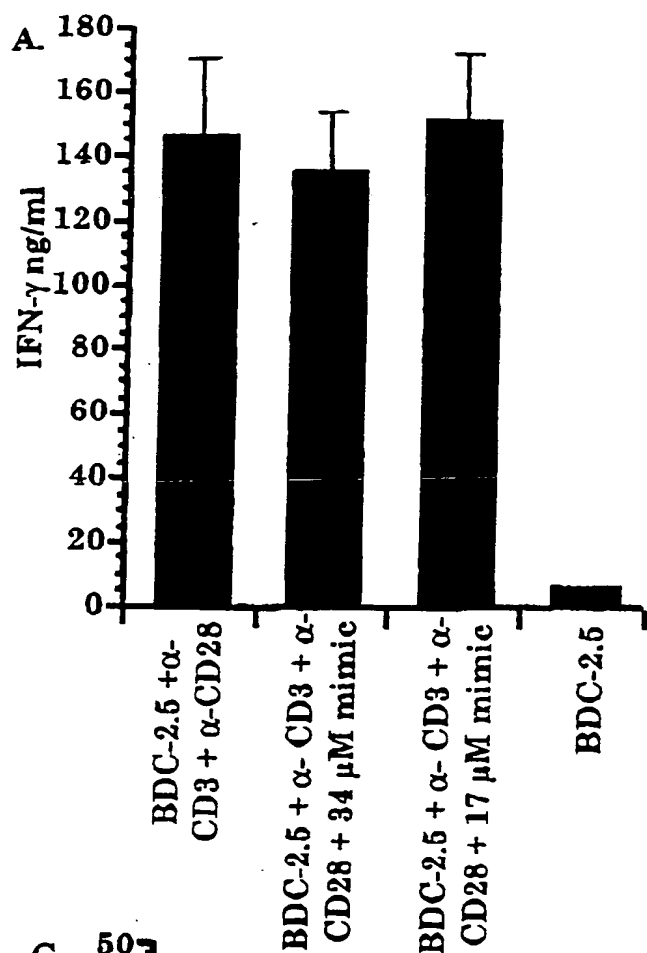
The present invention relates, in one embodiment, to a method of preventing or treating diabetes using low molecular weight antioxidants. In a further embodiment, the invention relates to a method of protecting and/or enhancing viability of cells/tissues/organs during isolation (harvesting), preservation, expansion and/or transplantation. In yet another embodiment, the present invention relates to a method of inducing immune tolerance. The invention also relates to compounds and compositions suitable for use in such methods.





**B.**





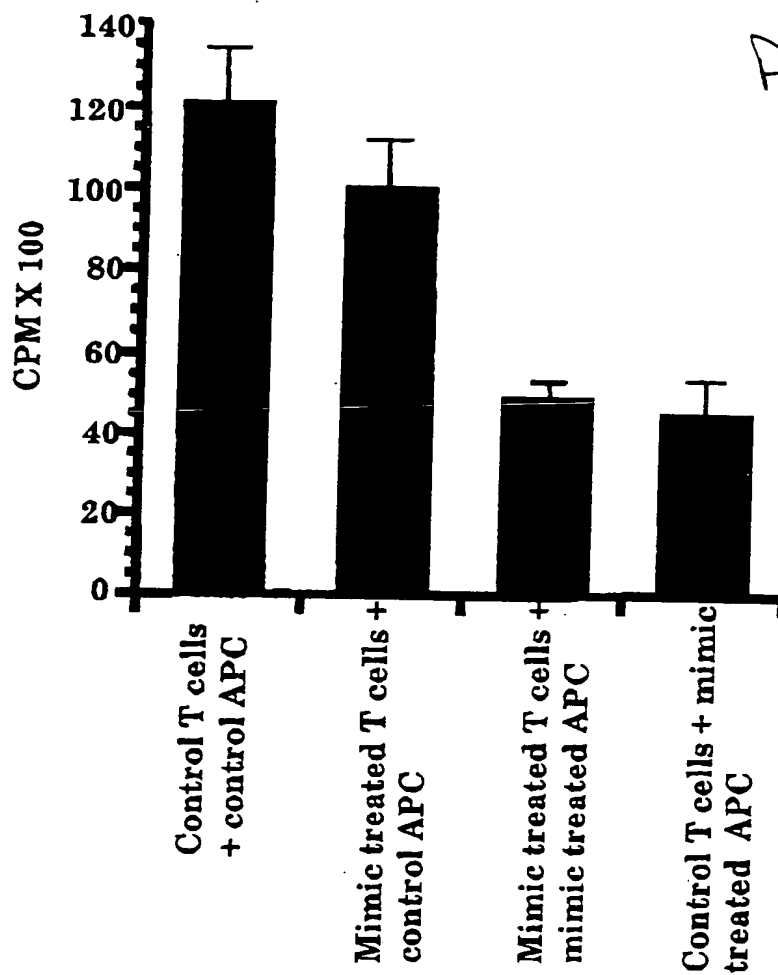
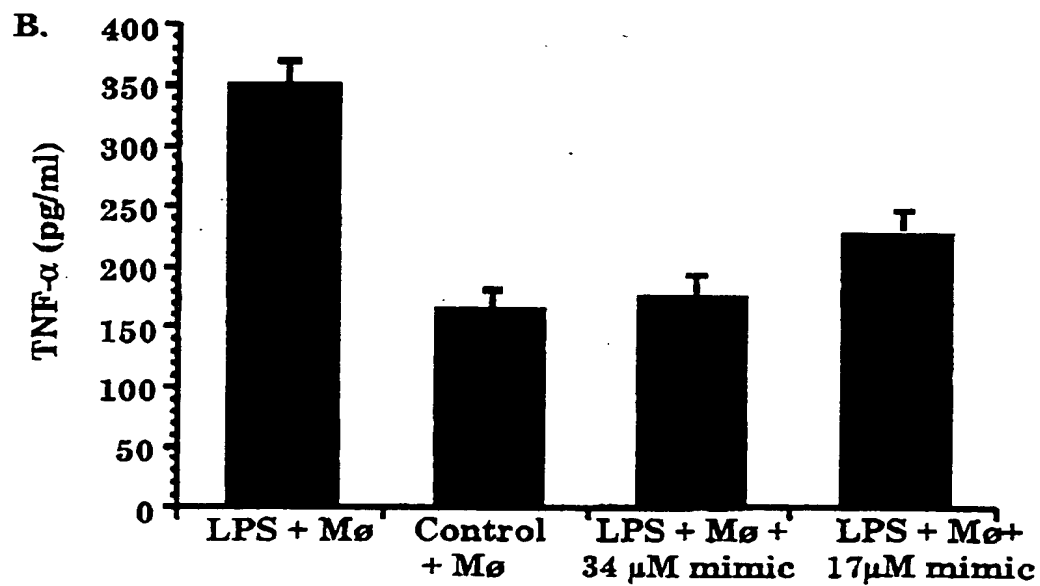
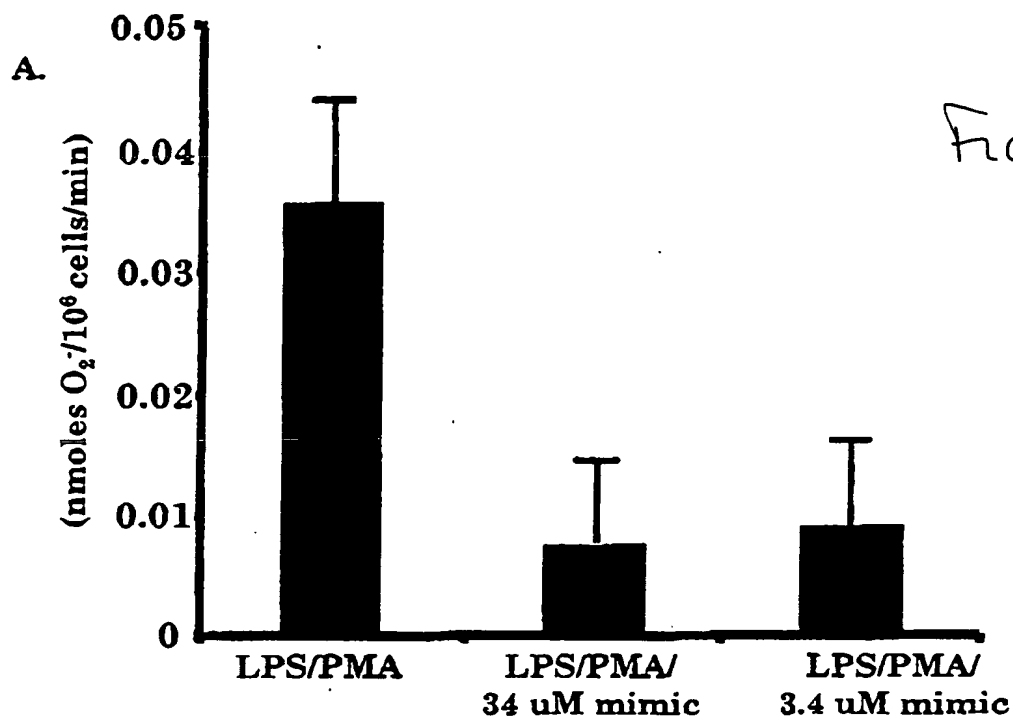


Figure 3



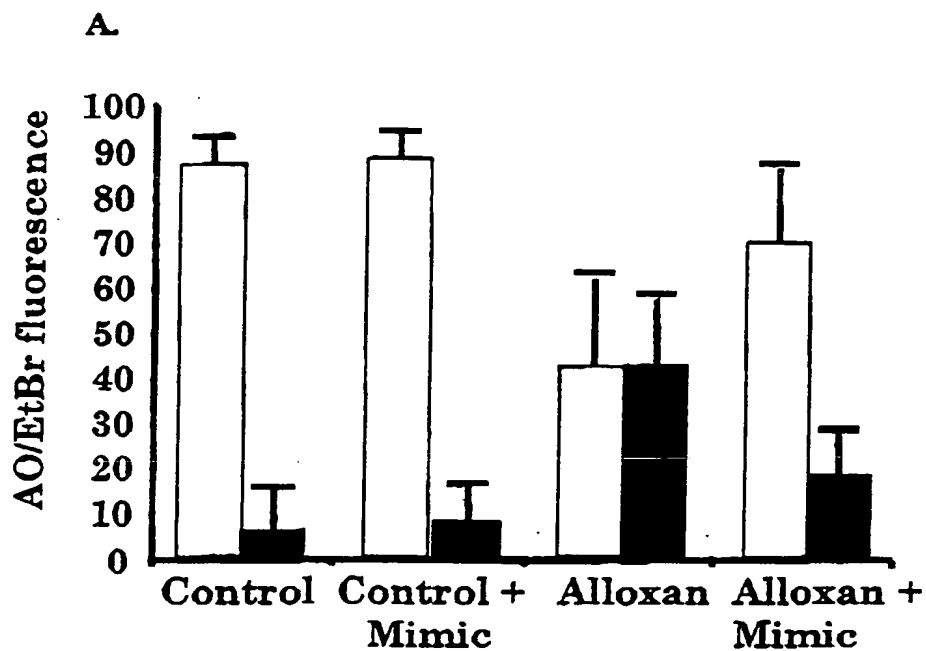


Figure 5

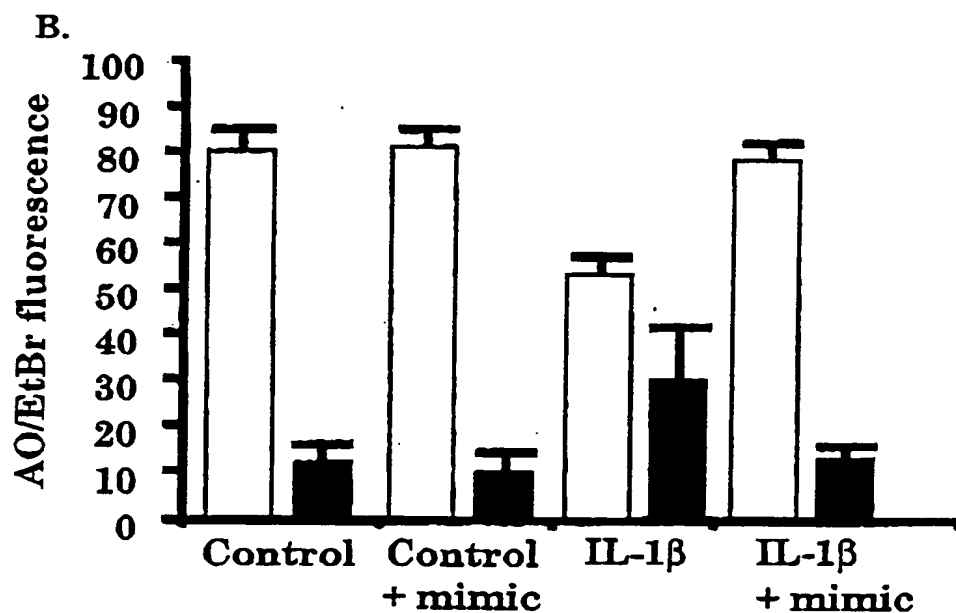


Figure 6

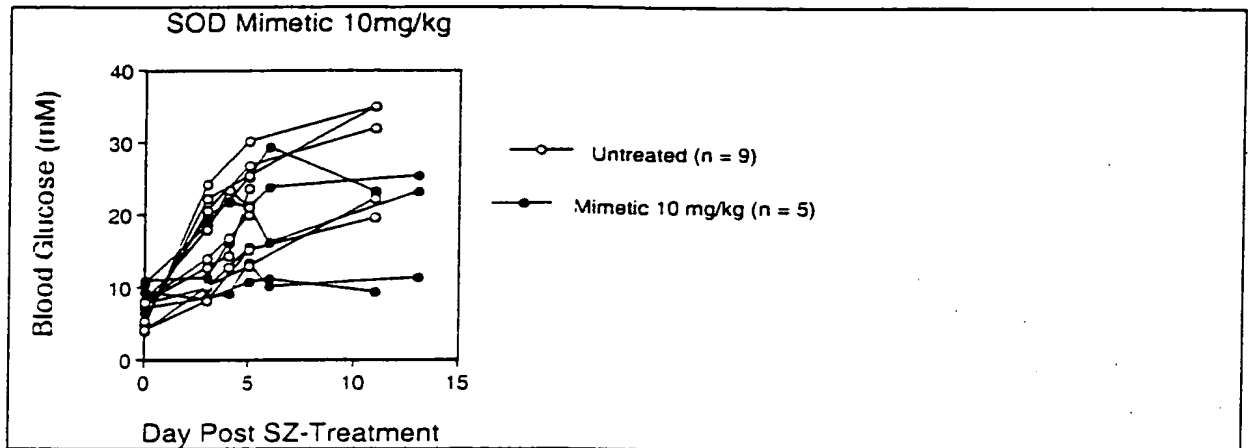


Figure 7

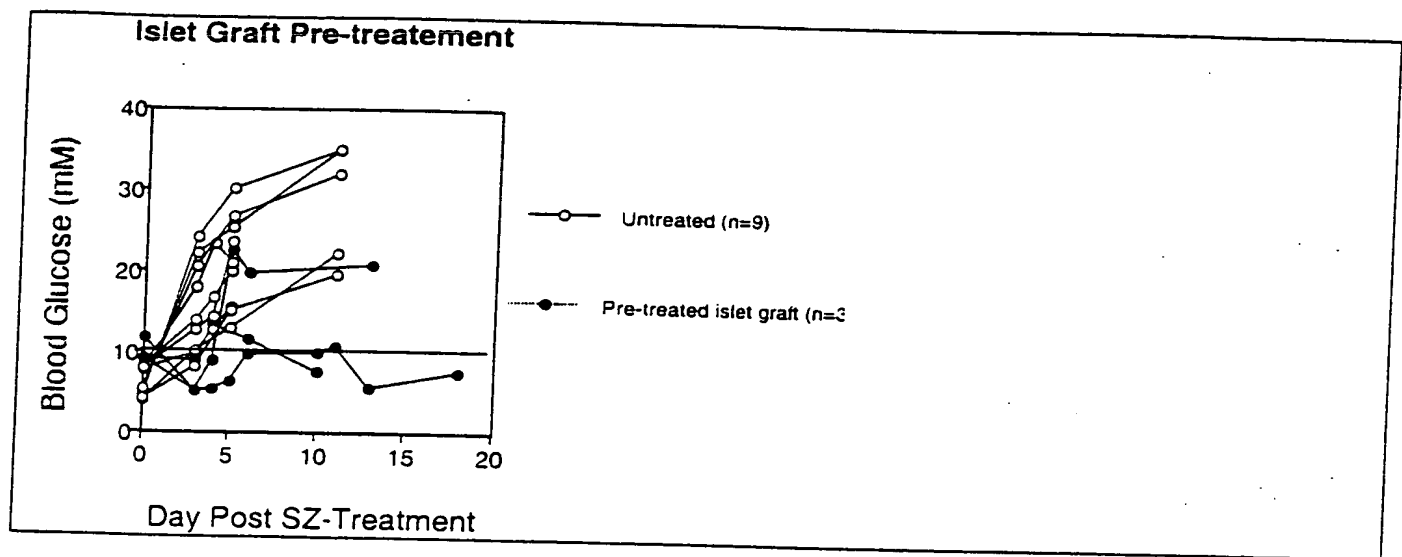


Figure 8

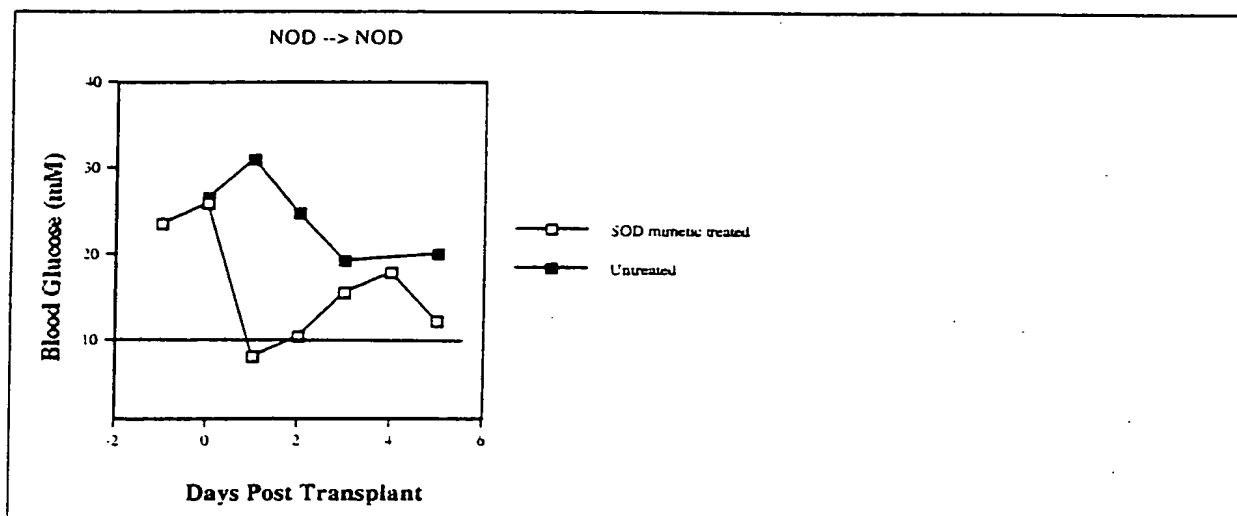
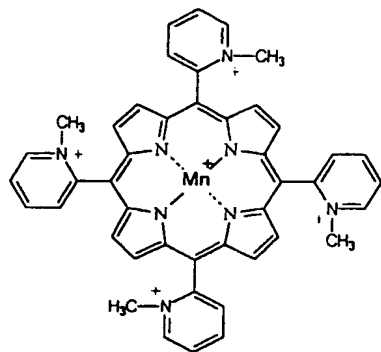
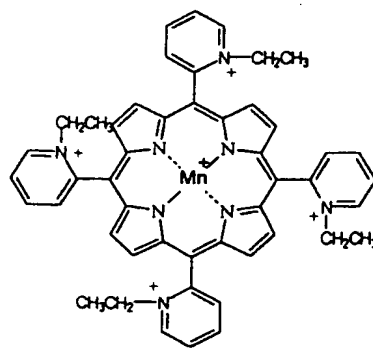




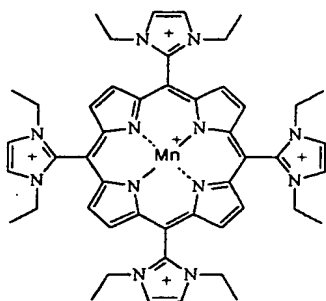
Figure 9



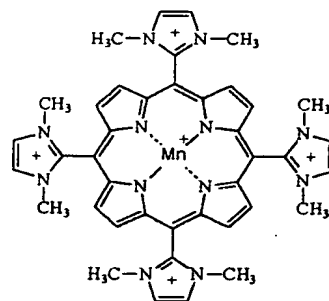
AEOL-10112



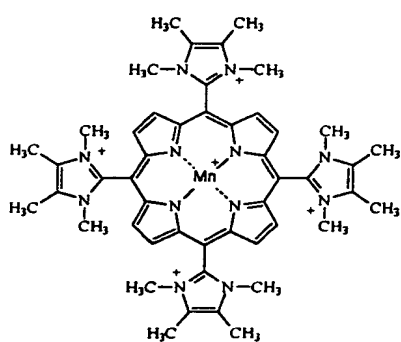
AEOL-10113



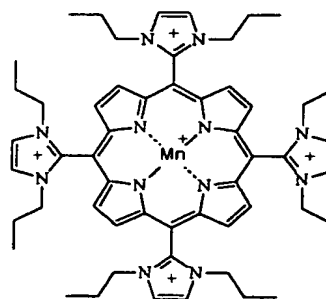
Aeol-10150



Aeol-10123



Aeol-10143



Aeol-10158